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Interactive effects of herbicide and enhanced UV-B on growth, oxidative damage and the ascorbate-glutathione cycle in two *Azolla* species

Sheo Mohan Prasad *, Sushil Kumar, Parul Parihar, Rachana Singh

Ranjan Plant Physiology and Biochemistry Laboratory, Department of Botany, University of Allahabad, Allahabad 211002 India

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ABSTRACT

A field experiment was conducted to investigate the impact of alone and combined exposures of herbicide pretilachlor (5, 10 and 20 μg ml^{-1}) and enhanced UV-B radiation (UV-B_1; ambient +2.2 kJ m^{-2} day^{-1} and UV-B_2; ambient +4.4 kJ m^{-2} day^{-1}) on growth, oxidative stress and the ascorbate-glutathione (AsA-GSH) cycle in two agronomically important *Azolla* spp. viz., *Azolla microphylla* and *Azolla pinnata*. Decreased relative growth rate (RGR) in both the species under tested stress could be linked to enhanced oxidative stress, thus higher H_2O_2 accumulation was observed, that in turn might have caused severe damage to lipids and proteins, thereby decreasing membrane stability. The effects were exacerbated when spp. were exposed to combined treatments of enhanced UV-B and pretilachlor. Detoxification of H_2O_2 is regulated by enzymes/metabolites of AsA-GSH cycle such as ascorbate peroxidase (APX) and glutathione reductase (GR) activity that were found to be stimulated. While, dehydroascorabte reductase (DHAR) activity, and the amount of metabolites: ascorbate (AsA), glutathione (GSH) and ratios of reduced/oxidized AsA (AsA/DHA) and GSH (GSH/GSSG), showed significant reduction with increasing doses of both the stressors, either applied alone or in combination. Glutathione-*S*-transferase (GST), an enzyme involved in scavenging of xenobiotics, was found to be stimulated under the tested stress. This study suggests that decline in DHAR activity and in AsA/DHA ratio might have led to enhanced H_2O_2 accumulation, thus decreased RGR was noticed under tested stress in both the species and the effect was more pronounced in *A. pinnata*. Owing to better performance of AsA-GSH cycle in *A. microphylla*, this study substantiates the view that *A. microphylla* is more tolerant than *A. pinnata*.

1. Introduction

Azolla, an aquatic fern has received special attention in recent years because of its demand as organic food, the source of protein for mono-gastric animals and biofertilizer (Raja et al., 2012). At field level, it has been reported that *Azolla* due to its quick decomposition and efficient nitrogen availability increases rice yield by 20–30% (Raja et al., 2012). Apart from this, it can cause rapid mineralization under water logged condition, increase water holding capacity, organic carbon, ammonium nitrogen and availability of phosphorus, potassium, calcium and magnesium of soil (Ram et al., 1994) and can also increase tolerance to salinity (Anjuli et al., 2004). It also has diverse application, viz. biofuel and bio-energy production and also contributes to human food, space diets and nutritional supplements to livestock (for more detail see review by Raja et al., 2012).

The changing agricultural practices worldwide have substantially resulted in increased dependency on several pesticides (Matson et al., 1997) and contamination caused by these pesticides has been very well reviewed by Huber et al. (2000) and Laabs et al. (2007). Pretilachlor is a chloroacetanilide herbicide (a group of pesticide) used in the rice fields to limit the growth of annual grasses, sedges, and many broad-leaved weeds. Further, biodegradability and the short half-life period ($t_{1/2}$) of this herbicide (Vidotto et al., 2004; Xie et al., 2004) argue for its increased use in cultivation of rice and several important food crops like soybean, maize, sugarcane, etc. (Huber et al., 2000; Laabs et al., 2007). However, several reports (Laabs et al., 2007; Konstantinou et al., 2006) have documented that due to lack of suitable microbial degraders, high alkalinity, and other conditions, these group of herbicides persist in soil and keep on accumulating. This group of herbicide inhibits vital processes in plants like seed germination, pigment and gibberellic acid synthesis, cell division, mineral

Abbreviations: APX, ascorbate peroxidase; AsA, reduced ascorbate; DHA, oxidized ascorbate (dehydroascorbate); DHAR, dehydroascorbate reductase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized form of glutathione; GST, glutathione-*S*-transeferase; MDA, malondialdehyde; ROS, reactive oxygen species

* Corresponding author.
E-mail address: profsmprasad@gmail.com (S.M. Prasad).

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uptake, disturbance in incorporation of amino acid into protein (Kearney and Kaufman, 1988) and major mode of action being suppression in protein synthesis (Chang et al., 1985) and very long-chain fatty acid (VLCFA) synthesis (Boger et al., 2000). Apart from this, repeated and indiscriminate use, careless handling, accidental spills and discharge of untreated effluents into water bodies has increased its concentration in the aquatic system.

In the natural field condition, the coexistence of more than one stressors are common. One such abiotic stress is solar UV-B radiation, which posses adverse effects on various biotic components of ecosystem primarily affecting the plant productivity that in turn may affect the ecological balance. UV-B radiation may negatively affect growth, development, morphology, physiology and biochemical processes of plants (Kataria et al., 2007). It may affect the plants directly by damaging DNA and membrane or indirectly by inducing the formation of reactive oxygen species, causing oxygen toxicity and thereby enhancing lipid and protein oxidation (Yannarelli et al., 2006).

In oxygenic photosynthetic organisms including *Azolla*, PS I and PS II as well as mitochondrial-mediated electron transport are the major sites of ROS ($O_2^{\cdot -}$,H_2O_2, ·OH, $^1O_2$) generation under normal as well as stressful conditions. However, the presence of toxic factors such as herbicide and high UV-B radiation enhances the generation of ROS through the same electron transport systems and thus accelerates chance of biomolecule damage (He and Häder, 2010; Sheeba et al., 2011). Thus, to prevent the cellular system from the damage caused by excessive ROS production, plants have evolved the antioxidant system. Among antioxidants, thioredoxins, peroxiredoxins and thioredoxin reductases as well as components of ascorbate-glutathione (AsA-GSH) cycle play a prominent role to control/detoxify the ROS especially H_2O_2 (Noctor and Foyer, 1998; Dietz et al., 2002; Kirchsteiger, et al. 2009) and mainly operates in chloroplast whereby AsA-GSH cycle regulates its function. This cycle is comprised of three independent redox couples: reduced and oxidized ascorbate ratio (AsA/DHA), reduced and oxidized glutathione ratio (GSH/GSSG), and NADPH/$NADP^+$ ratio (Noctor and Foyer, 1998; Drazkiewicz et al., 2003) and enzymes i.e., ascorbate peroxidase (APX; EC 1.11.1.11), glutathione reductase (GR; EC 1.6.4.2), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) and dehydroascorbate reductase (DHAR; EC 1.8.5.1) (Noctor and Foyer, 1998; Drazkiewicz et al., 2003). In the first step, APX which is most important H_2O_2 detoxifying enzyme (k_m value in μM range) than CAT and POD (k_m value in mM range) is reduced by ascorbate producing two oxidized products i.e. MDHA and DHA that are converted back to AsA by MDHAR and DHAR using the electron donors, NADPH and GSH. GR another important enzyme that in combination with APX metabolizes H_2O_2 into H_2O by reducing GSSG into GSH using NADPH as reducing power. Thus, possible alteration in the AsA-GSH cycle may affect the proper scavenging of H_2O_2, which could have an adverse effect on growth and over-all physiology of the organism. Glutathione-*S*-transferase (GST, EC 2.5.1.18), another important antioxidant that plays important role in detoxifying herbicide/ROS induced products by catalyzing conjugation reaction between herbicides/toxic radicals and GSH (Marrs, 1996), and toxic radical/herbicide-GSH conjugate formed are less toxic than herbicide/toxic radical molecules (Edwards et al., 2000) and thereby reducing the further damage caused to cell.

Thus, owing to the importance of AsA-GSH cycle and GST under stress condition, in the present study, effects of pretilachlor together with UV-B radiation on changes in the enzymes of the AsA-GSH cycle and related metabolites were investigated. As in earlier findings (Prasad et al., 2016), we noticed *Azolla microphylla* was more tolerant than *Azolla pinnata*, therefore the present piece of work is an extension to evaluate the mechanism of tolerance between the two *Azolla* spp. (*A. microphylla* and *A. pinnata*) by analysing efficiency of AsA-GSH cycle with reference to its enzymes and metabolites.

2. Materials and methods

2.1. Plant material and growth conditions

Two species of *Azolla*, viz. *Azolla pinnata* and *Azolla microphylla* were used for present study. *A. pinnata* was collected from the pond of Roxburgh garden, Department of Botany, University of Allahabad, while *Azolla microphylla* was procured from National Center for Conservation and Utilization of Blue Green Algae, IARI, New Delhi, India. The plants were treated with mercuric chloride (0.1% for 30 s) solution to surface sterilize the *Azolla* fronds. Thereafter, fronds were washed several time gently with sterile distilled water and finally placed in plastic trays ($32 \times 25 \times 6\ cm^{-3}$) containing combined-N free medium (Espinase and Watanabe, 1976). Prior to this, the pH of the medium was adjusted to 7.2. Plastic trays were placed in open field, and during the experimental period the temperature was found to vary from 16.7 to 36.8 °C, relative humidity from 55% to 71% and photosynthetic active radiation (PAR) from 800 to 1000 μmol photon $m^{-2}\ s^{-1}$.

2.2. Herbicides and UV-B treatments

The selected herbicide, pretilachlor [2-chloro-2′, 6′-diethyl-*N*-(2-propoxyethyl) acetanilide] 50% EC was used at three doses i.e. 5, 10 and 20 μg $ml^{-1}$and two levels of enhanced UV-B radiation, the low (UV-B_1: 2.2 kJ m^{-2} day^{-1}) and high (UV-B_2: 4.4 kJ m^{-2} day^{-1}) along with ambient were selected for detailed study. The dose selection of herbicide and enhanced UV-B radiation was based on screening experiment as described in earlier finding (Prasad et al., 2016). Enhanced UV-B radiation was obtained by UV-B lamps (Q-Panel Co, UV-B-313 fluorescent lamps, OH, USA), fixed perpendicular to fronds kept in the plastic trays on an adjustable frame. Samples were exposed to enhanced UV-B radiation daily during 9:30 (3.5 h after the beginning of the photoperiod) to 15:30 h. Each sample in open field was receiving ambient level (8.6 kJ m^{-2} day^{-1}) of UV-B along with enhanced UV-B radiation i.e. UV-B_1 and UV-B_2. In order to remove all incident UV-C (< 280 nm), radiation was filtered through 0.127 mm cellulose acetate (Johnston Industrial Plastics, Toronto, Canada). With the help of power meter (Spectra physics, USA Model 407, A-2) UV-B irradiance under the lamp at the surface of fronds was measured. All the parameters were analyzed after 96 h of treatment.

2.3. Growth analysis

Growth in treated and untreated samples of both the *Azolla* spp. was measured in terms of relative growth rate, calculated using equation:

$$RGR\left(day^{-1}\right) = (\ln W_2 - \ln W_1)/(t_2 - t_1)$$

where, W_1 is initial fresh mass (mg), W_2 is final fresh mass (mg), T_1 represent the initial time and T_2 final time for experiment.

2.4. Estimation of hydrogen peroxide (H_2O_2) content and indices of oxidative damage

Hydrogen peroxide (H_2O_2) content in treated and untreated *Azolla* fronds was estimated following the method of Velikova et al. (2000). Lipid peroxidation was determined by the method of Heath and Packer (1968) by measuring production of

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